IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Francesco Saverio DIOGUARDI	
Serial No.	:	10/575,062	Examiner: Christina Bradley
Cnfrm. No.	:	6565	Art Unit: 1654
Filed	:	April 7, 2006	
Title	:	AMINO-ACID COMPOSITIONS FOR THE TREATMENT OF PATHOLOGICAL CONDITIONS DISTINGUISHED BY INSUFFICIENT MITOCHONDRIAL FUNCTION	

DECLARATION OF ENZO NISOLI
UNDER 37 C.F.R. § 1.132

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, ENZO NISOLI, pursuant to 37 C.F.R. § 1.132, declare:

1. I received a degree in Medicine from the Brescia University in Italy and a Ph. D. in Pharmacology from the Brescia University in Italy.

2. I currently hold the position of Associate Professor of Pharmacology and Pharmacotherapy at the School of Medicine, Milan University.

3. Attached hereto as **Exhibit A** is a copy of my current Curriculum Vitae.

4. I have reviewed and am familiar with the subject matter described and currently claimed in the above-identified patent application having U.S. Serial No. 10/575,062 (also referred to herein as "U.S. Application No. 10/575,062," the "subject patent application," and the "subject application").

5. I have reviewed and am familiar with the rejections contained in the Final Office Action (mailed October 27, 2009) currently pending in the subject patent application.

6. I have also reviewed and understand the following documents:

(a) U.S. Patent Publication No. US 2007/0010437 A1, which is the published version of the subject patent application;

(b) Italian Patent Application No. TO 2003A000789, which is the patent application from which the subject patent application claims priority;

(c) Amendment submitted by applicant on June 22, 2009 in the subject patent application, which Amendment includes the currently pending claims;

(d) Final Office Action mailed by the Examiner on October 27, 2009 in the subject patent application;

(e) U.S. Patent No. 6,218,420 to Dioguardi (referred to herein as "Dioguardi '420" or "Dioguardi"); and

(f) U.S. Patent Publication No. US2004/0157903 to Conti et al.

7. I am presenting this Declaration to provide scientific reasoning, scientific evidence, and experimental results to contradict and rebut the currently pending obviousness (35 U.S.C. § 103(a)) and nonstatutory obviousness-type double patenting rejections based on Dioguardi '420.

8. As described in more detail in the following paragraphs, this Declaration supports the view that it would not have been obvious or routine in view of Dioguardi '420 to arrive at the molar ratios currently recited in independent claims 32 and 43. Further, determining the molar ratios recited in claims 32 and 43 of the subject patent application was not simply a result of optimization of the amounts and ratios of amino acids in the composition described in Dioguardi '420. Instead, making such molar ratio determinations yielded unexpected results that required extensive experimentation. The molar ratios recited in the claims are critical to the methods of independent claims 32 and 43.

9. Claim 32 of the subject application recites a method for maintaining intact, restoring, and/or increasing the number of cellular mitochondria in an elderly subject. Claim 43 of the subject application recites a method of treatment of apoptosis of mitochondrial origin.

10. With regard to claims 32 and 43, the Examiner makes the following statement:

> The limitation in claim 32 regarding maintaining intact, restoring and/or increasing the number of cellular mitochondria and the limitation in claim 43 regarding the treatment of apoptosis of mitochondrial origin, would necessarily be present in the method taught by Dioguardi because the composition, manner of administrating it and patient population are present in the prior art.

(Final Office Action, at page 5, the second full paragraph). As set forth in more detail below, scientific reasoning or evidence does not support these conclusions by the Examiner.

11. A key to understanding the distinctions between the teachings of Dioguardi and the claims of the subject patent application is to understand the cycle of nitrogen in the human body. Nitrogen is a specific component of proteins (which are the structural elements of human body), and should be introduced daily in correct quantities to maintain the integrity of the human body and to match the daily nitrogen losses. Nitrogen daily losses can be attributed to different causes, including, for example: (i) transformation of proteins to carbohydrates and lipids because of specific body needs; (ii) utilization of part of protein intake for energy purposes, for example, for balancing inadequate glucose availability, and/or (iii) intake of incorrect proportions of amino acids (contained in food) to specific human needs with consequent occurring of high nitrogen disposal loads. Thus, integrity of the human body depends on an adequate nitrogen intake.

12. Dioguardi refers to compositions based on amino acids for preventing and treating alimentary overloads in conditions of elevated body nitrogen requirements. In particular, Dioguardi describes diet compositions comprising specific amino acids in specific relative ratios that (a) enhance maintenance of the equilibrium between synthesis and degradation of proteins, (b) reduce the risks of under nutrition for some amino acids, and/or (c) reduce the risk of overload of those amino acids less useful to nitrogen metabolism, still maintaining easily under control the caloric input and at the same time reducing the load on disposal system of the body by minimizing catabolic products (i.e., urea, uric acid, etc.), without altering calcium excretion.

13. By way of contrast, the invention claimed in U.S. Application No. 10/575,062 concerns a ***completely different*** field of medicine, even though some similarities with the composition disclosed by Dioguardi can be observed. U.S. Application No. 10/575,062 deals with the problem of ***insufficient mitochondrial function***. Mitochondria are membrane-enclosed organelles found in most eukaryotic cells. Mitochondria generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of chemical energy. In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling, cellular differentiation, and cell death, as well as the control of the cell cycle and cell growth. Mitochondria are implicated in several human diseases, including mitochondrial disorders and cardiac dysfunction, and play a role in the aging process.

14. The most prominent roles of mitochondria are to produce ATP (i.e., via phosphorylation of ADP) through respiration, and to regulate cellular metabolism. The central set of reactions involved in ATP production are collectively known as the citric acid cycle. The production of ATP is done by oxidizing the major products of glucose, pyruvate, and NADH. This process of oxidation is dependent on the presence of oxygen, and is also known as cellular aerobic respiration. In the citric acid cycle done by mitochondria, each pyruvate molecule (produced by glycolysis) is actively transported across the inner mitochondrial membrane, and into the matrix where it is oxidized and combined with coenzyme A to form CO_2, acetyl-CoA, and NADH. The acetyl-CoA is the primary substrate to enter the citric acid cycle. The citric acid cycle oxidizes the acetyl-CoA to carbon dioxide, and, in the process, produces reduced cofactors (NADH and $FADH_2$) that are a source of electrons for the electron transport chain, and a molecule of guanosine triphosphate, which is readily converted to an ATP. During the citric acid cycle a small percentage of electrons may prematurely reduce oxygen, forming reactive oxygen species (ROS), which can cause oxidative stress in the mitochondria and may contribute to the decline in mitochondrial function associated with the aging process.

15. In view of the above, in one aspect, the invention of U.S. Application No. 10/575,062 is based on the discovery by applicant that the administration to a mammal of specific amino acid compositions characterized by specific relative ratios of amino acids may play an important role in survival of cells and the very duration of the life of cells, since the administration of these compositions plays a role in the mitochondria life cycle and is primarily able to increase the number of mitochondria, and/or restore normal mitochondrial function.

16. To illustrate the effectiveness of the invention claimed in the subject patent application, and to provide further evidence of the distinction of the claimed invention over Dioguardi, various experiments were designed and performed. These experiments and their results are described below, and were all performed under my direction.

17. Mitochondrial dysfunction due to oxidative damage is a major contributor to aging and age-related disorders, including loss of muscle mass and cardiovascular diseases. It will be herein demonstrated that a specific branched-chain amino acid mixture induces mitochondrial biogenesis in cardiac myocytes.

18. Figures 1, 2, and 3 presented below summarize experimental results relating to the invention claimed in the subject patent application.

19. Figure 1 shows that treatment of HL-1 adult cardiomyocytes with the amino acid mixture of U.S. Application No. 10/575,062 increases the mRNAs encoding mitochondrial biogenesis proteins, including peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), nuclear respiratory factor-1 (NRF-1), mitochondrial DNA transcription factor A (Tfam), and β-subunit of the mitochondrial H^+-ATP synthase (β-F1-ATPase). Mitochondrial gene targets of PGC-1α involved in oxidative phosphorylation, such as cytochrome c oxidase subunit IV (COX IV) and cytochrome c (Cyt c), were also upregulated by the amino acid mixture of U.S. Application No. 10/575,062 (Fig. 1B). These effects on gene expression translated into a 2.6-fold increase in mtDNA content (Fig. 1C), a 2-fold increase in citrate synthase activity (Fig. 1D), and a 1.5-fold increase in ATP amount (Fig. 1E) by amino acids.

20. These results strongly suggest that the amino acid mixture of U.S. Application No. 10/575,062 is able to increase energy production in cardiomyocytes. Remarkably, these results were not observed in C2C12 myotubes, nor L6 myoblasts treated with amino acids (Figure 2). Unlike the latter's, HL-1 cells spontaneously contract *in vitro*, suggesting that the amino acid effect on mitochondrial biogenesis in muscle cells is linked to contractile activity. Moreover, either white and brown adipocytes or 3T3-L1 fat cells were insensitive to amino acids (Figure 3).

FIGURE 1

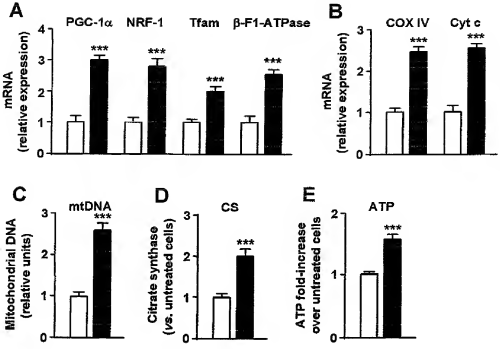

Figure 1 (caption): Amino acids promote mitochondrial biogenesis in HL-l cardiomyocytes. (A) PGC-lα, NRF-1, Tfam, β-Fl-ATPase, (B) COX IV, and Cyt c mRNA analysed by means of quantitative RT-PCR. The cycle number at which the transcripts were detectable was compared to that of 18S rRNA and expressed as relative values, with those measured in the untreated cells (open bars) taken as 1.0 ($n = 5$ experiments; $^{***}P < 0.001$). (C) Mitochondrial DNA amount, analyzed by means of quantitative PCR. The relative units are expressed in comparison to those of untreated cells (open bars) taken as 1.0 ($n = 5$ experiments; $^{***}P < 0.001$). (D) Citrate synthase activity and (E) ATP levels in cardiomyocytes. The values are expressed as fold-change vs. untreated cells (open bars) taken as 1.0 ($n = 5$ experiments; $^{***}P < 0.001$).

FIGURE 2

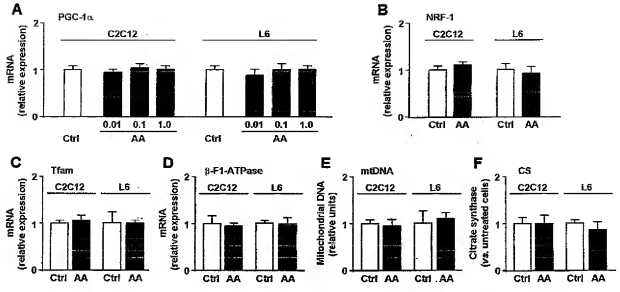

Figure 2 (caption): C2C12 myotubes and L6 myoblasts are insensitive to amino acids in promoting mitochondrial biogenesis. Final 0.0 1X to 1X amino acids (AA) concentrations were used (1X concentration when not specified). (A to D) PGC-1α, NRF-1, Tfam, and β-Fl-ATPase mRNA analysed by means of quantitative RT-PCR. The cycle number at which the various transcripts were detectable was compared to that of 18S rRNA and expressed as relative values, with those measured in the untreated cells (open bars) taken as 1.0 (*n* = 5 experiments). (E) mtDNA amount, analyzed by means of quantitative PCR and expressed as mtDNA copy number per nuclear DNA copy number, with values of the untreated cells (open bars) taken as 1.0 (*n* = 5 experiments). (F) Citrate synthase activity. The values are expressed as fold changes *vs.* untreated cells taken as 1.0 *(n* = 3 experiments). All data represent mean ± SEM.

FIGURE 3

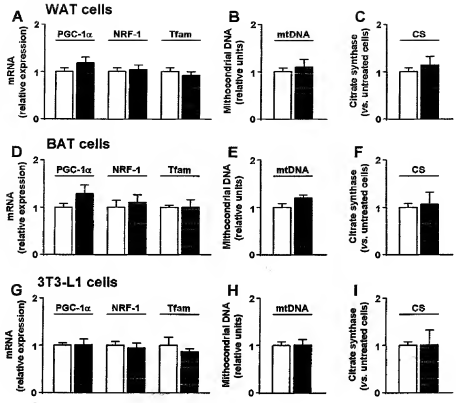

Figure 3 (caption): Primary white (WAT) and brown (BAT) adipose cells as well as 3T3-Ll adipocytes are insensitive to amino acids in promoting mitochondrial biogenesis. Final 1X amino acid concentration was used. (A, D, G) PGC-lα, NRF-l, Tfam mRNA analysed by means of quantitative RT-PCR. The cycle number at which the various transcripts were detectable was compared to that of 18S rRNA and expressed as relative values, with those measured in the untreated cells (open bars) taken as 1.0 *(n* = 5 experiments). (B, E, H) mtDNA amount, analyzed by means of quantitative PCR and expressed as mtDNA copy number per nuclear DNA copy number, with values in the untreated cells (open bars) taken as 1.0 *(n* = 5 experiments). (C, F, I) Citrate synthase activity. The values are expressed 3S fold change *vs.* untreated cells (open bars) taken as 1.0 *(n* = 5 experiments). All data represent mean ± SEM.

21. Taken together, the results presented herein demonstrate that oral administration of the amino acid mixture of U.S. Application No. 10/575,062 increases mitochondrial biogenesis in cardiac cells. This is an unexpected result in view of Dioguardi.

22. ***HL-l cell culture and treatment.*** HL-l, a cell line derived from adult mouse heart, were a gift from Dr. W.C. Claycomb (Louisiana State University, New Orleans, LA). Cardiomyocytes plated in flasks coated with 5 µg/ml fibronectin (Sigma Aldrich, Milan, Italy) and 0.02% gelatin (Fisher Scientific, Pittsburgh, P A) were maintained in Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 100 µM norepinephrine [from a 10mM norepinephrine (Sigma Aldrich, Milan, Italy) stock solution dissolved in 30 mM L-ascorbic acid (Sigma Aldrich, Milan, Italy)], 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen, San Giuliano Milanese, Italy) and 10% fetal bovine serum (FBS, JRH Biosciences) (complete medium). During culture, the medium was changed routinely every 48 hours. For experiments with amino acids, cells were grown to 70-80% confluence, and treated with the amino acid mixture of U.S. Application No. 10/575,062 freshly diluted in complete medium at final concentration 0.1X and 1X for 48 hours. Medium osmolarity was measured with an OM-6050 Osmo Station (Menarini, Florence, Italy): complete medium, 304 ±3 mOsm/kg; medium with 0.1X of the amino acid mixture of U.S. Application No. 10/575,062, 305 ± 2 mOsm/kg; medium with 1X of the amino acid mixture of U.S. Application No. 10/575,062, 349 ± 4 mOsm/kg. Exposure of HL-l cells to sucrose to produce a final medium osmolarity of 350 ± 3 mOsm/kg did not affect mtDNA content nor expression of mitochondrial biogenesis markers.

23. ***Cell culture.*** C2Cl2 myotubes and L6 myoblasts (American Type Culture Collection, Manassas, VA) were maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (growth medium). Cells were incubated at 37°C in humidified air containing 5% CO_2. C2C12 myoblasts were differentiated in DMEM with 2% horse serum. 3T3-Ll preadipocytes (American Type Culture Collection, Manassas, VA) were maintained at 37°C in 5% CO_2 in DMEM containing 4.5 g/L glucose, 4 mM glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 10% FBS. For differentiation, the cells were grown to confluence, and then maintained in the same medium for two more days before switching to the same medium plus 250 nM insulin, 0.5 mM IBMX and 1.0 µM dexamethasone. After 2 days, the medium was replaced by a medium containing 250 nM insulin, and

differentiation was allowed to proceed for until day 10-12. The undifferentiated and differentiated cells were treated or not with the amino acid mixture of U.S. Application No. 10/575,062, 305 for 48 hours, then harvested for further analysis. Brown and white fat precursor cells were enzymatically isolated from the interscapular BAT and epididymal WAT of wild-type mice, which were kept in standard laboratory conditions (12 h light/dark cycle; food and water ad libitum) as previously described (Nisoli E. et al., Science 299: 896-899, 2003; Tedesco L. et al., Diabetes 57: 2028-2036, 2008). The results obtained with differentiated cells were shown.

24. ***Quantitative Reverse Transcription (RT)- PCR.*** Total RNA was extracted from cells or tissues using the RNeasy® Lipid (or Fibrous) Tissue Mini Kit (Qiagen, Milan, Italy) and subjected to on-column DNAse digestion according to manufacturer's instructions. 1 µg of total RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Milan, Italy). Primers were designed using Beacon Designer 2.6 software from Premier Biosoft International (Palo Alto, CA, USA). Triplicate PCR reactions were carried out with the intercalating dye SybrGreen. Each sample (25 µl) contained 12.5 µl iQ™ SybrGreenl SuperMix (Bio-Rad), 0.4 µM each primer and 1/50 of reverse transcriptase product. PCR cycles were programmed on an iCycler iQ™ Real Time PCR detection system (Bio-Rad). The cycle number at which the various transcripts were detectable (threshold cycle, CT) was compared to that of 18S rRNA, referred to as ΔCT. The gene relative level was expressed as $2^{-(\Delta\Delta CT)}$, in which ΔΔCT equals ΔCT of the treated cells minus ΔCT of the untreated cells.

25. ***Mitochondrial DNA analysis.*** Mitochondria were isolated from tissue and cells. The mtDNA was extracted and analyzed according to the following protocol. Briefly, an aliquot of n1tDNA was loaded on ethidium bromide-stained agarose gel (1.2%) and quantified using the QuickImage densitometer (Packard). To determine mtDNA levels, the signal intensities were normalized to cell numbers in experiments on cultured cells or to protein content in experiments using tissues from wild-type and knockout mice. Alternatively, total DNA was extracted with QIAamp DNA extraction kit (Qiagen, Milan, Italy) following manufacturer's instructions. Mitochondrial DNA was amplified using primers specific for the mitochondrial cytochrome B (CytB) gene and was normalized to genomic DNA by parallel amplification of the large ribosomal protein p0 (36B4) nuclear gene.

1. ***ATP measurement.*** The whole amount of ATP in cells was measured by using the ATP determination kit from Molecular Probes. Cells were harvested by trypsinization and centrifuged at 500 g for 5 min at 4°C. ATP was extracted by incubating cell pellets with 1% trichloroacetic acid/4 mM EDTA solution for 10 min on ice. Cell extracts were centrifuged at 12,000 g for 10 min at 4°C and used for ATP determination as indicated by the manufacturer.

2. ***Citrate synthase activity.*** The activity was measured spectrophotometricaly at 412 nm at 30 °C in whole cell extracts. Tissue or cell homogenates were added to buffer containing 0.1 mM 5,5-dithio-bis-2-nitrobenzoic acid, 0.5 mM oxaloacetate, 50 µM EDTA, 0.31 mM acetyl CoA, 5 mM triethanolamine hydrochloride, and 0.1 M Tris-HCl, pH 8.1. Citrate synthase activity (measured as nmol citrate produced/min/mg protein) was expressed as the mean ± SEM of the fold change relative to the control set at 1.0 value.

3. ***Statistical analysis.*** Raw data from each experiment were normalized, combined, and analysed using either t test or one-way analysis of variance with Tukey multiple comparison post hoc test. A probability of 5% was considered significant. Statistical analyses were performed using GraphPad Prism® (version 4.0 GraphPad Software7 La Jolla7 CA,USA).

4. From the above reported test it is evident that the amino acid based compositions employed in the presently claimed method for maintaining intact, restoring, and/or increasing the number of cellular mitochondria in elderly subject and for the treatment of apoptosis of mitochondrial origin is effective in that it provides an increase in mitochondrial biogenesis and would not have been expected by one of skill in the art in view of Dioguardi

5. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

March 5th, 2010
Date

Enzo Nisoli